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Patentanmeldung Nr. Patent application No. Demande de brevet n°

99104033.8

## PRIORITY DOCUMENT

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**Blatt 2 der Bescheinigung  
Sheet 2 of the certificate  
Page 2 de l'attestation**

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69115 Heidelberg  
GERMANY

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Use of TGF-inhibitors for treating cerebral disorders

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Applicant: Biopharm Gesellschaft zur biotechnologischen Entwicklung von  
Pharmaka mbH

"Use of TGF-inhibitors for treating cerebral disorders"

EPO - Munich

Our Ref: B 1931EU - py / js

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16. März 1999

### Description

The present invention relates to the use of a compound capable of substantially inhibiting the biological activity of TGF- $\beta$  on predamaged neurons, for treating cerebral disorders, and to pharmaceutical compositions containing said compound and a second compound for disintegrating blood clots.

The transforming growth factor  $\beta$  (TGF- $\beta$ ) family contains subspecies TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3, which are widely distributed and contextually acting cytokines with prominent roles in development and cell cycle control (Roberts and Sporn, 1990; Kriegelstein et al., 1995). TGF- $\beta$ s have been implicated in the regulation of neuronal survival of e.g. motoneurons, sensory and midbrain dopaminergic neurons. The TGF- $\beta$  isoforms show widely overlapping expression patterns. Nullmutations for each of these isoforms show distinct phenotypes, which, however, are restricted to non-neural tissues (Shull et al., 1992; Kaartinen et al., 1995; Proetzel et al., 1995; Sanford et al., 1997). Mice deficient for the TGF- $\beta$  receptor type-II (T $\beta$ R-II) are lethal at E 10.5, i.e. prior to the development of the nervous system.

During the development of the vertebrate nervous system, large numbers of neurons in the central and peripheral nervous system undergo naturally occurring cell death (Oppenheim, 1991). Regulation of neuron survival or death, respectively, requires the concerted actions of sets of molecules, which act cell-extrinsically and -intrinsically to execute or prevent cell death (Pettmann and Henderson, 1998).

Cerebral disorders such as a neurodegenerative disorder or cerebral ischaemiae, result in injury or death of neurons in mammals, and produce motor and/or

cognitive deficits that are often permanent. At present, in most of these cerebral disorders there is no treatment that reliably improves the prognosis of a patient suffering from said disorders.

5 Thus, the technical problem underlying the present invention is to provide a new system imparting protection and therefore survival on predamaged or injured neurons upon a certain cerebral disorder.

10 The solution of the above technical problem is achieved by providing the embodiments characterized in the claims.

In particular, the present invention is based on the fact that, beside promoting intact neurons, TGF- $\beta$  is the major regulator for the execution of predamaged neurons upon a certain cerebral disorder. Accordingly, the present invention  
15 relates to the use of a compound capable of substantially inhibiting the biological activity of TGF- $\beta$  on predamaged neurons, for treating cerebral disorders in mammals, preferably in man. The term "TGF- $\beta$ " comprises the subspecies TGF- $\beta$ 1, TGF- $\beta$ 2, and TGF- $\beta$ 3.

20 In a preferred embodiment of the present invention the term "compound capable of substantially inhibiting the biological activity of TGF- $\beta$  on predamaged neurons" refers to polyclonal or monoclonal antibodies directed to TGF- $\beta$ , as TGF- $\beta$  inhibitors, or to antagonists such as compounds having the binding site of a TGF- $\beta$  receptor, e.g. a TGF- $\beta$  RII/Fc chimeric protein (T $\beta$ R-II-Fc), as well as to  
25 low molecular weight compounds such as chemical or non-proteinaceous compounds, as TGF- $\beta$ -antagonists.

The cerebral disorder includes peripheral and/or CNS-disorders including cerebral and focal ischaemias such as apoplexy, and neurodegenerative disorders such as  
30 ALS. For example, adverse consequences of central nervous system injuries may be caused by thrombus, embolus, systemic hypotension, hypertension, hypertensive cerebral vascular disease, rupture of an aneurysm, an angioma, blood dyscrasias, cardiac failure, cardiac arrest, cardiogenic shock, septic shock, head

trauma, spinal cord trauma, seizure, bleeding from a tumor, or other blood loss. The spinal cord, which is also a part of the central nervous system, is equally susceptible to ischemia resulting from diminished blood flow.

- 5 Where the ischemia is associated with a "stroke", it can be either global or focal ischemia, as defined below.

10 By "focal ischemia", as used herein in reference to the central nervous system, is meant the condition that results from the blockage of a single artery that supplies blood to the brain or spinal cord, resulting in the death of all cellular elements (pan-necrosis) in the territory supplied by that artery.

15 By "global ischemia", as used herein in reference to the central nervous system, is meant the condition that results from a general diminution of blood flow to the entire brain, forebrain, or spinal cord, which causes the death of neurons in selectively vulnerable regions throughout these tissues. The pathology in each of these cases is quite different, as are the clinical correlates. Models of focal ischemia apply to patients with focal cerebral infarction, while models of global ischemia are analogous to cardiac arrest, and other causes of systemic hypoten-  
20 sion.

25 A further aspect of the present invention relates to a pharmaceutical composition containing, in pharmaceutically effective amounts, the above defined compound, and a second compound for disintegrating blood clots, and optionally a pharmaceutically acceptable carrier and/or diluent. In a preferred embodiment of the present invention, the second compound is selected from the group consisting of urokinase, thrombin, and tPA (tissue plasminogen activator).

30 The treatment regimen is carried out, in terms of administration mode, timing of the administration, in dosage, so that the functional recovery of the patient from the adverse consequence of the cerebral disorder is improved.

The administration of the compound or pharmaceutical composition according to

the present invention can be carried out by any standard route and known rule of administration, including intravenously, orally, or intracerebrally. The dosage of such antibodies or antagonists according to the present invention lies preferably within a range of circulating concentrations that include the ED<sub>50</sub> with little or no toxicity. The dosage can vary within this range depending upon the dosage form employed and the route of administration utilized.

The formulation of the above defined compound or the pharmaceutical composition according to the present invention does not exhibit any specific restriction, and may be prepared e.g. in the form of tablets, suppositories, solutions, or retarded release-formulations. The antibodies or antagonists for example can be formulated for parenteral administration by injection, for example, by bolus injection or continuous infusion. Formulations for injection can be presented in unit dosage form, for example, in ampules or in multi-dose containers, with an added preservative.

The therapeutic antibodies or antagonists of the invention can also contain a carrier or excipient and/or diluent, many of which are known to skilled artisans. Excipients which can be used include buffers (for example, citrate buffer, phosphate buffer, acetate buffer, and bicarbonate buffer), amino acids, urea, alcohols, ascorbic acid, phospholipids, proteins (for example, serum albumin), EDTA, sodium chloride, liposomes, mannitol, sorbitol, and glycerol.

It is well known in the medical arts that dosages for any one patient depend on many factors, including the general health, sex, weight, body surface area, and age of the patient, as well as the particular compound to be administered, in the time and route of administration, and other drugs being administered concurrently. Determining the most appropriate dosage and route of administration is well within the abilities of a skilled physician.

The figures show:



Fig. 1: Specificity of anti-TGF- $\beta$ -antibody as well as morphology and neuron numbers of chick ganglia at E10, i.e. after the main period of ontogenetic ciliary neuron death. Embryos were treated daily from E6 to E9 with 10  $\mu$ g of a monoclonal mouse anti-TGF- $\beta$ 1, - $\beta$ 2, - $\beta$ 3 antibody (anti-TGF- $\beta$ ; obtained from Genzyme) applied to the chorionic allantoic membrane, an identical dose of a mouse IgG with or without rhodamine conjugation, or 2  $\mu$ g of a recombinant human TGF- $\beta$  RII/Fc chimeric protein (T $\beta$ R-II-Fc; obtained from R&D Systems). Ciliary ganglia were dissected at E10, fixed in Bouin's solution, and paraffin-embedded. (A) Digitalized photograph of a dot blot showing the specificity of the anti-TGF- $\beta$ -antibody. (B) Photographs of sections (H.E. stain or fluorescence microscopy) through the largest circumference of ganglia from embryos with the indicated treatments. Bar = 100  $\mu$ m. (C) Graphic representations of neuron counts in ganglia with the indicated treatments. Data are given as mean  $\pm$  SEM ( $n=5$ ),  $p$  values derived from Student's t-test are \*\*\* $p<0.001$  for increased neuron numbers as compared to untreated embryos.

Fig. 2: Neuron numbers and apoptosis in ciliary ganglia (CG), dorsal root ganglia (L3; DRG), and the lumbar motoneuron column of embryos treated with anti-TGF- $\beta$ . (A) Graphic representation of neuron counts at E10 following daily treatments from E6 - E9. (B) Photographs of TUNEL labelings of CG, DRG, and lumbar motoneurons at E8 following treatment with anti-TGF- $\beta$ -antibody at E6 and E7 (lower panel) as compared to untreated embryos (upper panel). Arrowheads point at TUNEL-positive nuclei. (C) Graphic representation of a quantitative analysis of TUNEL-positive neurons. Data in (A) and (C) are given as mean  $\pm$  SEM [ $n=5$  in (A),  $n=3$  in (C)],  $p$  values derived from Student's t-test are \*\* $p<0.01$ , \*\*\* $p<0.001$  for increased (A) or decreased (C) neuron numbers as compared to untreated embryos.

Fig.3: Graphic representation of neuron counts showing phenotype rescue of CG and motoneurons in anti-TGF- $\beta$ -treated embryos by administration

of TGF- $\beta$ . Treatment with TGF- $\beta$ 3 (2 $\mu$ g) at E10 following administration of anti-TGF- $\beta$  from E6 to E9 (grey bars) produced neuron numbers at E14 (dashed bars) identical to control embryos (white bars). Continuing treatment with anti-TGF- $\beta$  from E10 to E13 (black bars) fully maintained neuron numbers. Data are given as mean  $\pm$  SEM ( $n=5$ ),  $p$  values derived from Student's  $t$ -test are  $**p<0.01$ ,  $***p<0.001$  for increased neuron numbers as compared to controls,  $+++p<0.001$  for decreased neuron numbers as compared to antibody-treated embryos.

Fig. 4: Qualitative microscopic and quantitative analyses showing that anti-TGF- $\beta$  rescues lumbar DRG and motoneurons following unilateral limb bud ablation. (A) Photographs of microscopic analyses of H.E. stained sections. Bar = 200  $\mu$ m. (B) Graphic representation of neuron counts at E7 following the indicated treatments. Data are given as mean  $\pm$  SEM ( $n=5$ ),  $p$  values derived from Student's  $t$ -test are  $***p<0.001$  for increased neuron numbers following limb bud ablation.

The present invention is illustrated by the following non-limiting example.

## EXAMPLE

### Neuron counting experiments

Chick embryos were treated daily with 50  $\mu$ l (phosphate buffered saline, supplemented with 200 units/ml penicillin, 100  $\mu$ g/ml streptomycin, 200  $\mu$ g/ml neomycin (each obtained from Gibco) containing either 10  $\mu$ g of a pan anti-TGF- $\beta$  (obtained from Genzyme) or 2  $\mu$ g T $\beta$ R-II-Fc (obtained from R&D Systems), or 10  $\mu$ g mouse IgG (obtained from Sigma) by administration onto the chorio-allantoic membrane through a window in the shell as described in Oppenheim et al., 1993. Embryos were killed on either E8, E10 or E14. The CG and the lumbar spinal cord with attached DRG were dissected, fixed in Bouin's solution and

paraffin embedded. All tissues were sectioned at 8 to 10  $\mu\text{m}$  and stained with H.E. Neurons were counted in every fifth or tenth section as described in Oppenheim et al., 1993.

## 5 TUNEL labeling

Sections were stained according to the manufacturer's instructions (Boehringer Mannheim) and counter-stained with H.E. TUNEL positive nuclei of large neurons were counted on every tenth section and expressed as ratio of total neuron of this particular neuron population.

## Unilateral limb bud ablation

Ablations of hind limb buds of chick embryos were performed at E3. Embryos were treated daily (10  $\mu\text{g}$ ; E3 - E6) as indicated above with either anti-TGF- $\beta$  or IgG and processed at E7 for H.E. staining and neuron counting.

## Anti-TGF- $\beta$ -antibody neutralizes endogenous TGF- $\beta$ during the main period of ontogenetic neuron cell death

In the developing nervous system of chick and mammals, TGF- $\beta$ 2 and - $\beta$ 3 as well as their receptors occur in many populations of postmitotic neurons, including CG, DRG, and motoneurons (Krieglstein et al., 1998). A monoclonal antibody recognizing all three isoforms of TGF- $\beta$  (anti-TGF- $\beta$ ) was used to neutralize endogenous TGF- $\beta$  during the main period of ontogenetic cell death (E6 - E9) of CG and DRG as well as spinal motoneurons. Ten  $\mu\text{g}$  of this antibody neutralize 10 ng of each available recombinant TGF- $\beta$  including chicken TGF- $\beta$ 3 (R&D Systems) to >98%, as assessed in a bioassay using mink lung epithelial cells (Abe et al., 1994; Krieglstein and Unsicker, 1995). The specificity of this monoclonal anti-TGF- $\beta$  antibody was ascertained by dot blot using 40 other growth factors (Fig. 1A). Fig. 1B shows that daily treatments with anti-TGF- $\beta$  resulted in an increased size of CG at E10. A corresponding control IgG, which is rhodamine-conjugated, could be detected throughout the entire embryo. A

TBR-II-Fc chimeric protein employed as an alternative tool to capture endogenous TGF- $\beta$  likewise increased the size of CG at E10 compared to untreated or IgG-treated embryos. Neuron counts (Fig. 1C) revealed that the increased size of CG is due to a significant increase in neuron numbers. The counted values of approximately 6,000 reflect neuron numbers prior to ontogenetic neuron death at E6 (Landmesser and Pilar, 1974) showing that elimination of TGF- $\beta$  signalling prevents the execution of ontogenetic neuron death in the chick CG.

### Neutralizing TGF- $\beta$ interferes with ontogenetic cell death in sensory and motoneurons

Sensory and motoneurons were analyzed to investigate whether neutralizing TGF- $\beta$  also interfered with the development of other neuron populations during the period of ontogenetic neuron death. Neuron counts in the L3 DRG and in the lumbar motoneuron column revealed a significant increase in neuron numbers at E10 following daily antibody treatments from E6 to E9 (Fig. 2A). Numbers of motoneurons in antibody-treated embryos almost matched neuron numbers prior to the onset of ontogenetic neuron death (Hamburger et al., 1975). To determine whether the increase in numbers of neurons upon anti-TGF- $\beta$ -treatment resulted from reduced ontogenetic neuron death, TUNEL labelling was performed to stain for apoptotic cells. Counting the numbers of TUNEL-positive cells at E8, which is the peak of cell death for all three neuron populations investigated, revealed a significant reduction of the proportion of apoptotic cells (Fig. 2B, C). This shows that endogenous TGF- $\beta$  is essential for executing the cell death program in developing neurons.

### Ontogenetic neuron death occurs after termination of antibody treatment and substitution of endogenous TGF- $\beta$

Furthermore, it was investigated whether ontogenetic neuron death would occur at a later time point following termination of the antibody treatment and substitution of endogenous TGF- $\beta$ . Fig. 3 shows that a single dose of TGF- $\beta$ 3 at E10 after the end of the antibody treatment (E6 -E9) resulted in a significant reduc-

tion of neuron numbers in the CG and motoneuron column. Numbers of neurons matched those of control embryos at the end of the main ontogenetic cell death period (E10) as well as embryos at E14. Therefore, TGF- $\beta$  mediates death of selected neurons, especially those that are destined to die. In contrast, prolonga-  
5 tion of the antibody treatment beyond the period of ontogenetic death stabilized neuron numbers at increased levels (Fig. 3).

#### **Treatment of limb bud-ablated embryos with anti-TGF- $\beta$ rescues both motoneurons and DRG neurons**

Multiple evidence suggests that the extent of ontogenetic neuron death is crucially regulated by the target (Olek and Edwards, 1978; Pittman and Oppenheim, 1979; Pilar et al., 1980; Thoenen and Edgar, 1985), best exemplified by the classic experiments of V. Hamburger (Hollyday and Hamburger, 1976).  
15 Extirpation of the hind limb bud in the E3 chick embryo, i.e. prior to the arrival and synapse formation of motoneuron axons in the target (Dahm and Landmesser, 1988, 1991), causes death of almost all lumbar motoneurons and sensory neurons by E7 (Oppenheim et al., 1978) (Fig. 4A). Qualitative microscopic analysis (Fig. 4A) documents that treatment of limb bud-ablated embryos  
20 with anti-TGF- $\beta$  rescues both motoneurons as well as DRG neurons. Quantification reveals that the lumbar motoneuron population in limb-ablated embryos could surprisingly be rescued by anti-TGF- $\beta$  treatment to approximately half the half the size as compared to the non-operated control side (Fig. 4B).

#### **Summary**

The above results show that TGF- $\beta$  exerts a key role both in the regulation of ontogenetic neuron death of three major classes of peripheral and CNS neurons as well as in the regulation of cell death following target ablation. Parasympa-  
30 thetic CG, sensory DRG, and spinal lumbar motoneurons in chick embryos have their main period of ontogenetic neuron death between E6 and E9, lose approximately 50% of the total cells generated, but require largely distinct and only partly overlapping target-derived trophic molecules to secure the survival of

optimal cell numbers. CG neurons are maintained by CNTF/GPA, DRG neurons mainly by neurotrophins, and motoneurons by GDNF, HGF, neurotrophins, IGF-I and others (Nishi, 1994; Heller et al., 1995; Lewin and Barde, 1996; Oppenheim, 1996). In motoneurons, even a cocktail of numerous trophic molecules cannot fully maintain the population. The example of the present invention demonstrates that the elimination of endogenous TGF- $\beta$  or TGF- $\beta$  signaling, respectively, results in rescuing effects, which are distinct from neurotrophic factor treatments in terms of (i) the broad spectrum of responsive populations and (ii) magnitude of effect, which encompasses virtually all neurons of a given population. Therefore, the lack of TGF- $\beta$  is able to rescue all neurons destined to die. The elimination of TGF- $\beta$  is superior to any of known manipulations of the molecular cascade involved in neuron cell death in that neuron populations are fully maintained.

The example shows that ontogenetic neuron death of ciliary, dorsal root and spinal motoneurons is largely prevented and neuron losses following limb bud ablation are greatly reduced following neutralization of endogenous TGF- $\beta$  by an anti-TGF- $\beta$  antibody in chick embryos. Likewise, preventing TGF- $\beta$  signaling by treatment with a T $\beta$ R-II fusion protein during the period of ontogenetic cell death in the ciliary ganglion rescues all neurons, which normally die. TUNEL staining revealed decreased numbers of apoptotic cells. Application of exogenous TGF- $\beta$  rescued the deprived phenotype. Thus, TGF- $\beta$  in contrast to any single neurotrophic factor acting in the above systems plays a key role in regulating ontogenetic neuron death as well as cell death following neuronal target deprivation.

The above results demonstrate the pivotal role of TGF- $\beta$  in regulating neuron survival and death. The data given above show that TGF- $\beta$  is an essential trigger of neuronal death and thus, compounds according to the present invention which are capable of inhibiting the biological activity of TGF- $\beta$  as e.g. TGF- $\beta$ -specific antibodies are exceptionally good therapeutic tools that prevent TGF- $\beta$  signalling in the treatment of stroke, neurotrauma, and neurodegenerative diseases.

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16. March 1999

Müller-Boré &amp; Partner

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Applicant: Biopharm Gesellschaft zur biotechnologischen Entwicklung von  
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"Use of TGF-inhibitors for treating cerebral disorders"

EPO - Munich  
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Our Ref: B 1931EU - py / js

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### Claims

1. Use of a compound capable of substantially inhibiting the biological activity of TGF- $\beta$  on predamaged neurons, for the preparation of a medicament for treating cerebral disorders.
- 5 2. Use according to claim 1, wherein said compound is an antibody directed to TGF- $\beta$  or a compound having the binding site of a TGF- $\beta$  receptor.
3. Use according to claim 1 or 2, wherein said disorder is a peripheral and/or CNS-disorder.
- 10 4. Use according to claim 3, wherein said disorder is a cerebral ischemia or a neurodegenerative disorder.
- 15 5. A pharmaceutical composition containing, in pharmaceutically effective amounts, a compound capable of substantially inhibiting the biological activity of TGF- $\beta$  on predamaged neurons, and a second compound for disintegrating blood clots, and optionally a pharmaceutically acceptable carrier and/or diluent.
- 20 6. The pharmaceutical composition according to claim 5, wherein said compound is an antibody directed to TGF- $\beta$  or a compound having the binding site of a TGF- $\beta$  receptor.
- 25 7. The pharmaceutical composition according to claim 5 or 6, wherein said second compound is selected from the group consisting of urokinase, thrombin, and tissue plasminogen activator.



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Fig. 1A

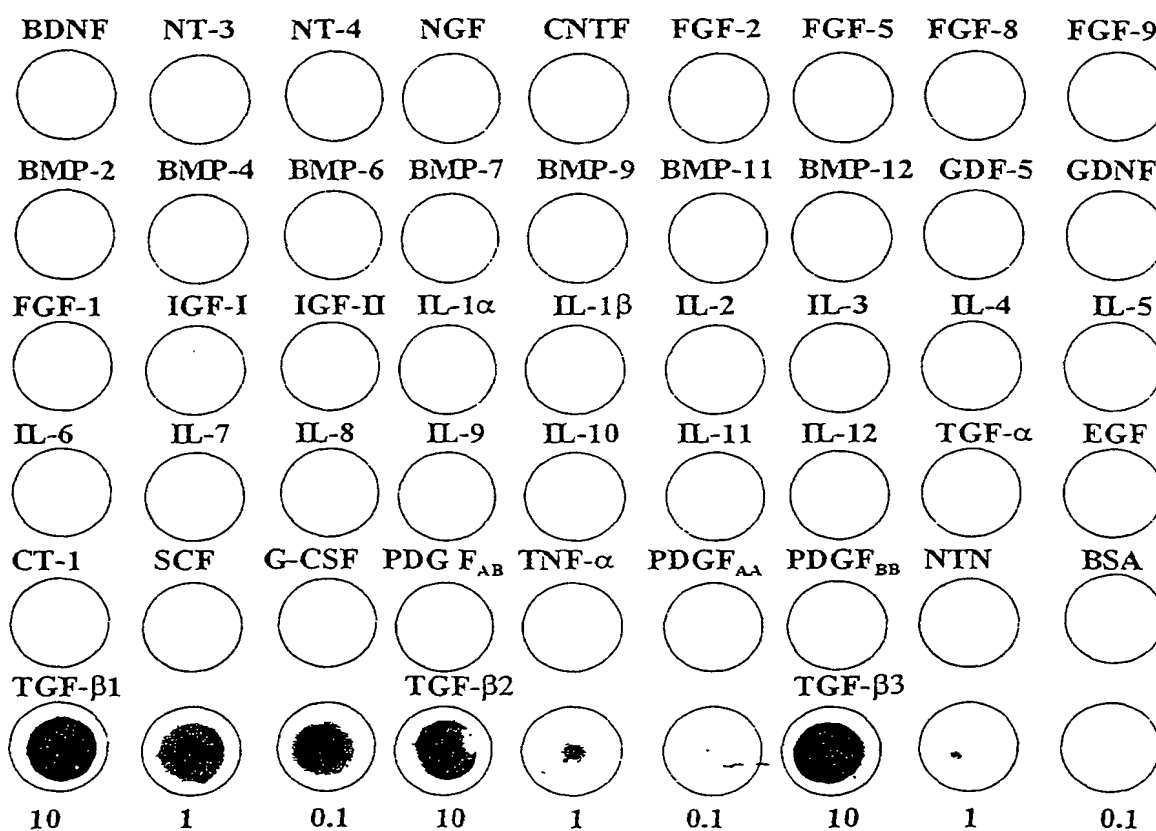
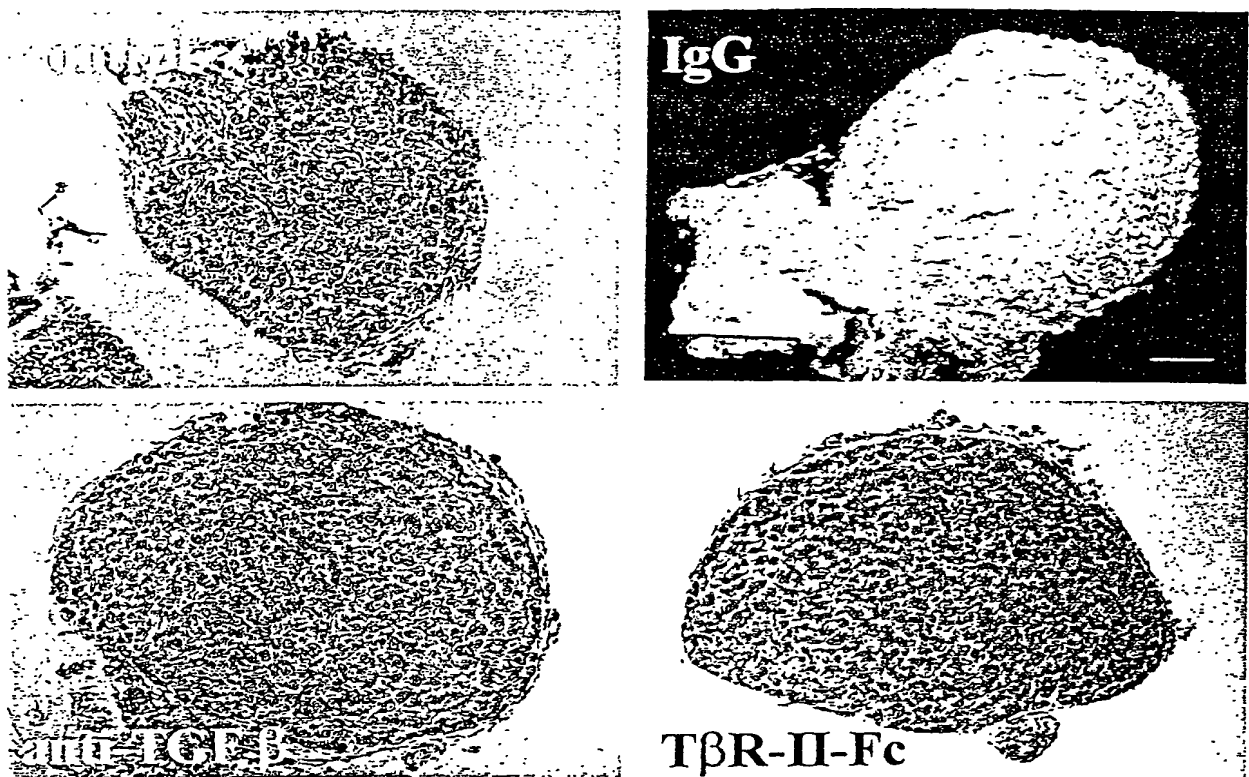
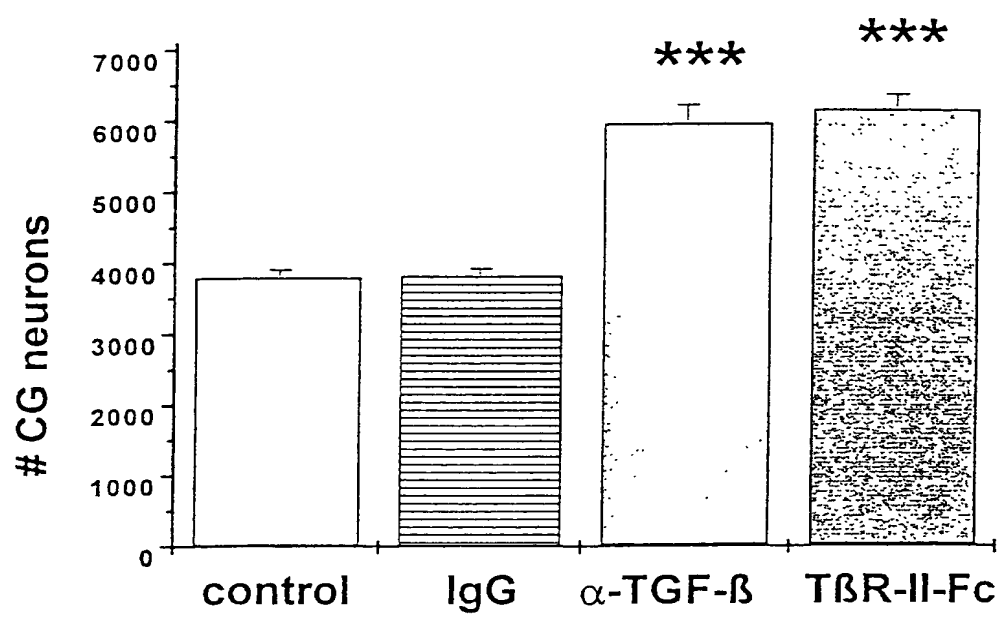


Fig. 1B



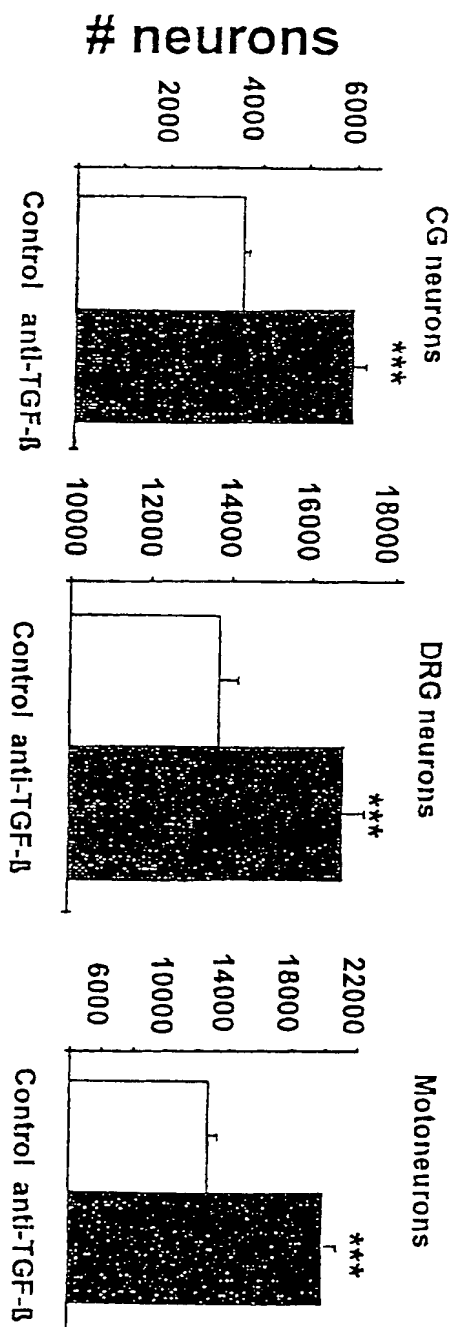
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Fig. 1C



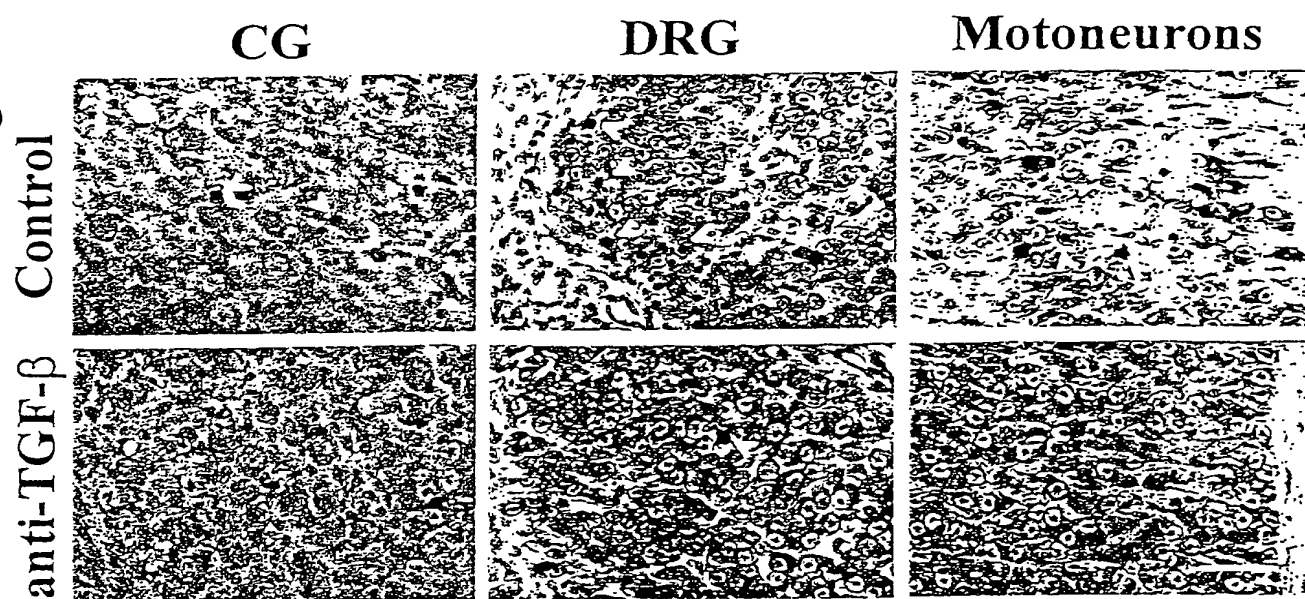
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Fig. 2A



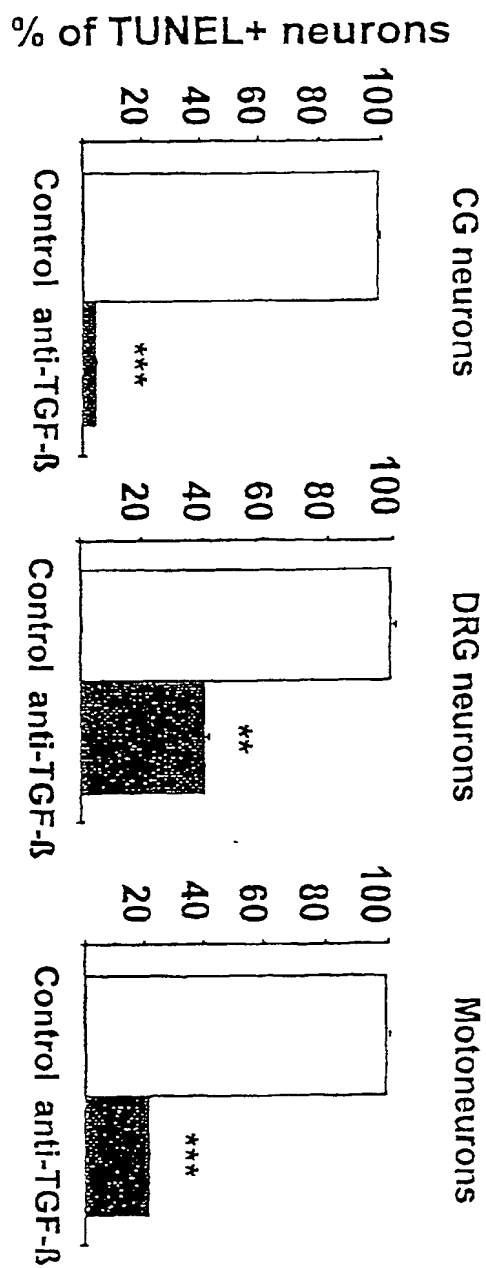
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Fig. 2B



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Fig. 2C





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Fig. 3

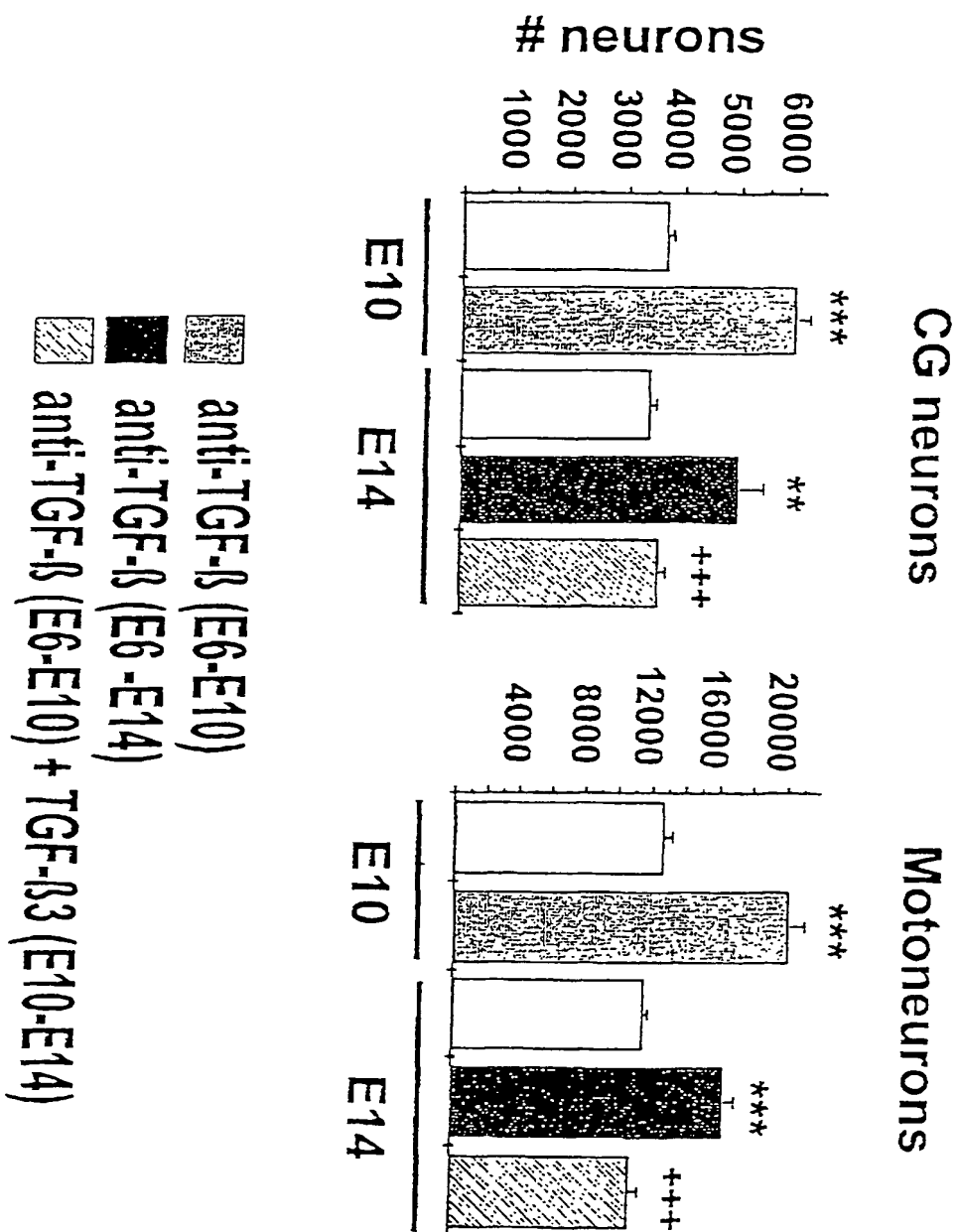
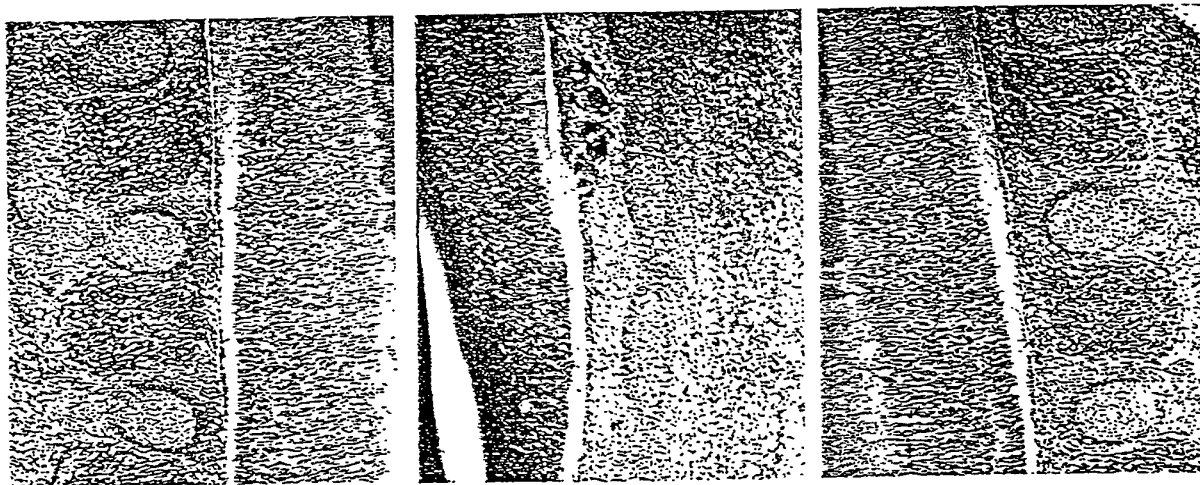


Fig. 4A

limb-ablated side

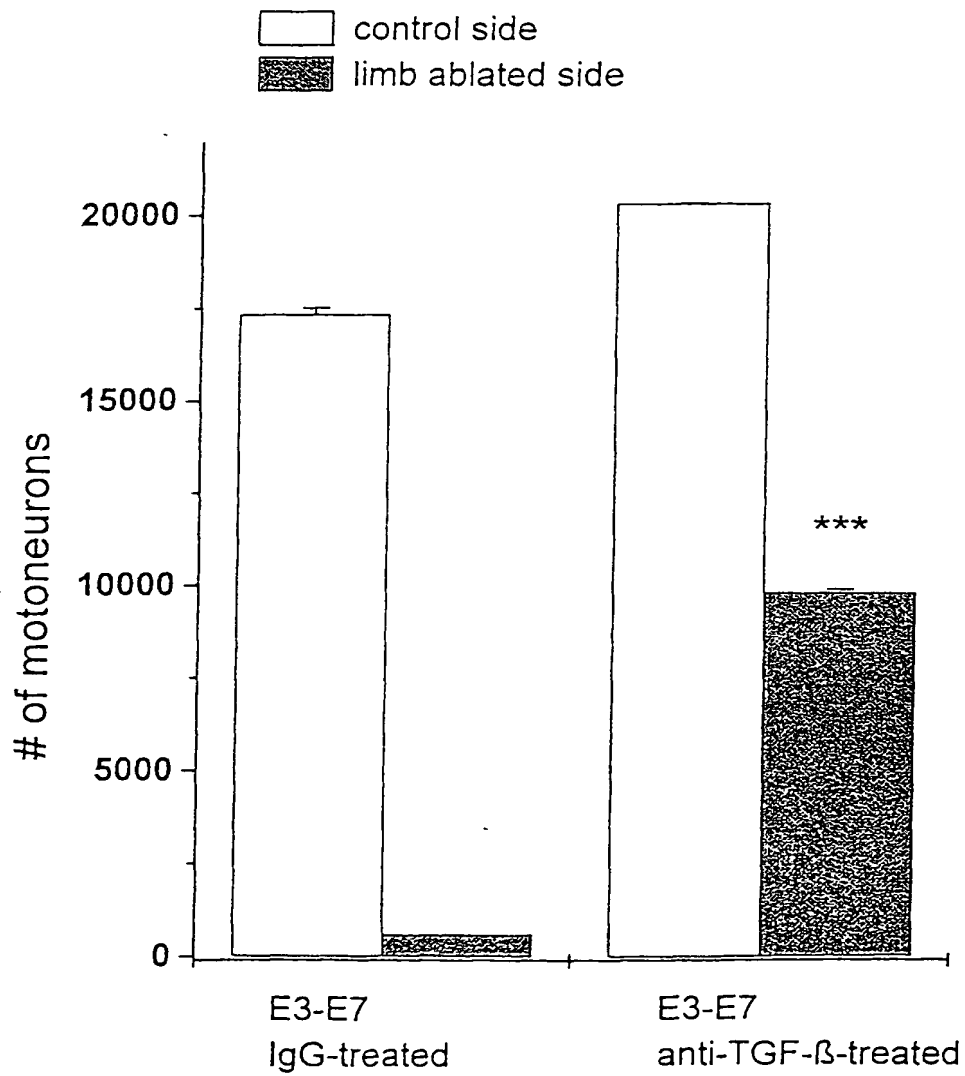


IgG-treated

anti-TGF- $\beta$  treated

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Fig. 4B





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### Abstract

The present invention relates to the use of a compound capable of substantially inhibiting the biological activity of TGF- $\beta$  on predamaged neurons, for treating cerebral disorders, and to pharmaceutical compositions containing said compound and a second compound for disintegrating blood clots.

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